Phospholipase A$_2$ Antagonists Inhibit Constitutive Retrograde Membrane Traffic to the Endoplasmic Reticulum

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Eukaryotic cells contain a variety of cytoplasmic Ca^{2+}-dependent and Ca^{2+}-independent phospholipase A$_2$s (PLA$_2$s; EC 2.3.1.2.3). However, the physiological roles for many of these ubiquitously-expressed enzymes is unclear or not known. Recently, pharmacological studies have suggested a role for Ca^{2+}-independent PLA$_2$ (iPLA$_2$) enzymes in governing intracellular membrane trafficking events in general and regulating brefeldin A (BFA)-stimulated membrane tubulation and Golgi-to-endoplasmic reticulum (ER) retrograde membrane trafficking, in particular. Here, we extend these studies to show that membrane-permeant iPLA$_2$ antagonists potently inhibit the normal, constitutive retrograde membrane trafficking from the *trans*-Golgi network (TGN), Golgi complex, and the ERGIC-53-positive ER-Golgi-intermediate compartment (ERGIC), which occurs in the absence of BFA. Taken together, these results suggest that iPLA$_2$ enzymes play a general role in regulating, or directly mediating, multiple mammalian membrane trafficking events.

Key words: Golgi complex, membrane tubules, phospholipase A$_2$, retrograde transport

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Researchers have employed a variety of biochemical, pharmacological, and genetic approaches to elucidate the physiological functions of cytoplasmic PLA$_2$s, enzymes that catalyze the hydrolysis of glycerophospholipids into lysophospholipids and free fatty acids [for reviews, see (1,2)]. The cytoplasmic PLA$_2$ fall into several distinct classes based on amino acid sequence similarities and include both Ca^{2+}-dependent (cPLA$_2$) and Ca^{2+}-independent (iPLA$_2$) enzymes (3).

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Although much is known about the biological functions of cPLA$_2$ in mammals, particularly their roles in arachidonic acid metabolism (4), much less is known about the physiological roles of the iPLA$_2$s. These enzymes include the ubiquitously expressed (Group VI) 80-kDa iPLA$_2$, and two (Group VII and VIII) iPLA$_2$s specific for platelet-activating factor (PAF) [for review, see (3)]. Fortunately, pharmacological reagents that antagonize both intracellular cPLA$_2$ and iPLA$_2$s, or are highly selective for only iPLA$_2$ enzymes, have provided investigators with tools to begin to explore the functions of these enzymes in living cells [for reviews, see (5,6)].

Recently, we have uncovered additional and unexpected roles for iPLA$_2$ enzyme activities in mammalian cells. Specifically, we have shown that low concentrations of a broad spectrum of membrane-permeant PLA$_2$ antagonists, including bromoenol lactone (BEL), a highly selective mechanism-based suicide substrate inhibitor of iPLA$_2$ enzymes, disrupt various intracellular membrane trafficking events that appear to depend on the formation of membrane tubules. These trafficking events include the membrane tubule-mediated, step-wise reassembly of Golgi complexes into an interconnected juxtanuclear ribbon (7) and, most relevant to this work, the tubule-mediated Golgi-to-ER retrograde trafficking that is induced by the fungal metabolite brefeldin A (BFA) (8).

In mammalian cells, BFA blocks ER-to-Golgi anterograde membrane trafficking and constitutive protein secretion by preventing the association of COPI and clathrin coat protein complexes with Golgi and TGN membranes, respectively (9). In addition, BFA causes the dramatic formation of long, thin membrane tubules that extend from the Golgi complex, TGN, and endosome membranes [for review, see (10)]. In the case of the Golgi complex, these tubules fuse with the ER, creating a hybrid Golgi-ER tubulovesicular compartment (11,12). TGN membranes also tubulate in the presence of BFA, but these membrane tubules fuse with early endosomal (EE) membranes, creating a hybrid TGN-EE tubulovesicular network (13,14). Interestingly, BFA does not disrupt the morphological integrity of the ER-Golgi-intermediate compartment (ERGIC), an organelle through which newly synthesized secretory proteins travel when trafficking from ER exit sites to the *cis*-Golgi proper (15). This last observation demonstrated that not all membrane-bound organelles in the early secretory pathway are BFA-sensitive.

A variety of biochemical, morphological, genetic, and pharmacological studies have now established the existence of robust recycling pathways from the Golgi complex and TGN back to the ER in normal cells (16–22). The remaining questions regarding retrograde trafficking center on identifying

the membranous intermediaries that mediate cargo transport, and the precise steps involved. For example, biochemical, morphological, and genetic studies have strongly implicated COPI-coated vesicles in one, or possibly several, steps in retrograde trafficking through the Golgi, although all of the exact ones have not been definitively identified (23,24). However, other membranous mediators of retrograde trafficking must also exist because recent studies have demonstrated COPI-independent retrograde trafficking as well (22,25). Indeed, membrane tubules have been strongly implicated in at least some step in Golgi-to-ER trafficking based on time-lapse imaging studies of green fluorescent protein (GFP)-labeled Golgi membrane proteins (26,27), and BFA-induced retrograde trafficking via membrane tubules (12–14).

Interestingly, BFA-stimulated Golgi membrane tubulation and retrograde membrane trafficking appear to require the activity of a cytoplasmic PLA$_2$ (7,8). For example, in PLA$_2$ antagonist-treated cells, Golgi membranes neither tubulated nor recycled back to the ER in response to BFA (8). In addition, potent peptide stimulators of intracellular PLA$_2$ enzymes, including melittin and a synthetic peptide derived from a melittin-like sequence contained within the PLA$_2$ Activating Protein (PLAP) (28), promoted the tubule-mediated retrograde trafficking of Golgi membranes back to the ER in a permeabilized, semi-intact cell system (29). These and other observations led to the hypothesis that PLA$_2$ antagonists directly interfered with the cellular membrane tubulation machinery and, in addition, that PLA$_2$ enzyme activities may be directly required for normal retrograde trafficking from the Golgi complex to the ER (8).

To investigate this possibility, we examined whether PLA$_2$ activity participates in the retrograde membrane trafficking that occurs in mammalian cells in the absence of BFA. More specifically, we examined three independent protein markers whose recycling from the Golgi to the ER can be studied in normal cells without the need for drug (BFA) induction: chimeric proteins of KDELR and VSV-G and TGN38 and VSV-G and ERGIC-53, a transmembrane lectin that cycles between the ER, the ERGIC, and *cis*-Golgi membranes (15,30). Here, we report that PLA$_2$ antagonists potently block all of these retrograde membrane trafficking events and, therefore, provide pharmacological evidence in support of the idea that intracellular calcium-independent PLA$_2$ enzymes play roles in each.

Results

To investigate the effects of membrane-permeant PLA$_2$ antagonists on anterograde and retrograde membrane trafficking between the ER and Golgi complex, we examined the thermo-reversible movement of two temperature-sensitive chimeric transmembrane glycoproteins, KDELR-G and TGN38-G, each of which was constructed by fusing the thermo-sensitive luminal domain of temperature-sensitive variant (ts045)-vesicular stomatitis virus G (VSV-G) fused to

the cytoplasmic and transmembrane domains of the KDELR (KDELR-G) and TGN38 (TGN38-G), respectively (20). These chimeras were constructed to take advantage of two separate domains: 1) the luminal domain of ts045 VSV-G, which undergoes a thermo-reversible conformational change at the restrictive temperature when in the ER that results in the inability of ts045 VSV-G to exit the ER (31); 2) organelle targeting and retention information contained within the cytoplasmic and transmembrane domains of KDELR and TGN38. At the permissive temperature (32°C) for VSV-G luminal domain folding, newly synthesized KDELR-G and TGN38-G fold and exit the ER to assume a normal steady-state distribution predominantly in the Golgi and TGN, respectively (20). However, following shift to the restrictive temperature (39.5°C), the Golgi- and TGN-associated levels of KDELR-G and TGN38-G decrease with a concomitant increase in the amounts of these proteins in the ER. Shift back to the permissive temperature allowed the chimeras to rapidly exit the ER and reassume a Golgi localization. Thus, these results demonstrate that both the chimeric proteins were constitutively cycling between the Golgi complex and the ER. We exploited this model system to analyze KDELR-G and TGN38-G trafficking in the presence of PLA$_2$ antagonists.

PLA$_2$ antagonists disrupt retrograde membrane trafficking from the Golgi and TGN to ER

When Clone 9 rat hepatocytes transiently transfected with plasmid DNA coding for the expression of KDELR-G or TGN38-G were maintained at the permissive temperature, the chimeric proteins were found by double-label immunofluorescence microscopy using monoclonal antibodies directed against VSV-G membrane glycoprotein epitopes colocalized to a large extent in the juxtanuclear region with the resident Golgi enzyme α-mannosidase II (Man II), as expected (Figure 1A,B; Figure 2A,B). On the other hand, when transfected cells were maintained at the permissive temperature and then shifted to the restrictive temperature for 1–3 h, a significant amount of KDELR-G and TGN38-G staining was shifted to a more diffuse and nuclear envelope pattern without affecting the distribution of Man II (Figure 1C; Figure 2C). In addition, under these conditions, the diffuse KDELR-G and TGN38-G colocalized with ERP60, an ER resident protein (Figure 1C,D; Figure 2E,F). These observations therefore indicated that the chimeric proteins had recycled from their downstream steady-state localizations in the Golgi complex and TGN back to, and accumulated within, the ER after the temperature shift.

Importantly, when cells were first pre-incubated (10 min) at the permissive temperature with the irreversible iPLA$_2$ antagonist BEL (1 μM), and then shifted for various periods of time to the restrictive temperature in the presence of this compound, KDELR-G and TGN38-G remained predominantly localized in the juxtanuclear Golgi region where Man II was found (Figure 1E,F; Figure 2G,H). Similarly, when cells were incubated continuously at the permissive temperature in the presence of BEL for 60 min, KDELR-G and TGN38-G remained in the juxtanuclear Golgi region (data not shown).

Quantifying these results revealed that following shift to the restrictive temperature in the absence of PLA$_2$ antagonists, 50–60% of the transfected cells were found to have detectable staining of KDELR-G and TGN38G in the nuclear envelope and ER after 1 h (Figure 3). We believe that the 50–60% value is probably an underestimate of the total percentage of cells in which KDELR-G accumulated in the ER because of the loss of signal when Golgi proteins recycle and accumulate in the ER. This loss of signal is especially significant in a population of transiently transfected cells exhibiting a wide spectrum of expression levels. Nevertheless, incubation of cells with ≥1 μM BEL significantly inhibited the redistribution of both KDELR-G and TGN38-G to the ER/nuclear envelope following shift to the restrictive temperature (Figure 3). Together, these observations suggest that Golgi-to-ER and TGN-to-ER retrograde trafficking, and the consequent accumulation of KDELR-G and TGN38-G, was significantly inhibited by the intracellular PLA$_2$ antagonist.
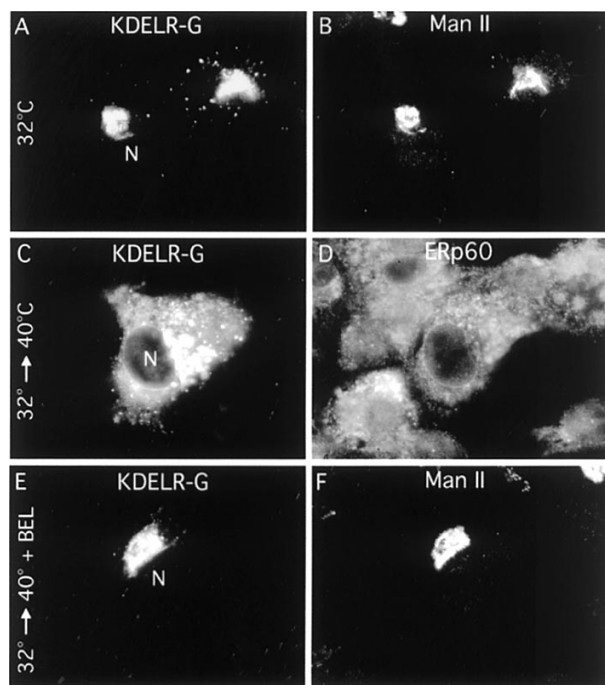

Figure 1: Inhibition of constitutive recycling of KDELR-G from the Golgi complex to the ER by the PLA$_2$ agonist, BEL. Double immunofluorescence of KDELR-G (A, C, E) and Man II (B, F) of ERp60 (D) is shown. A, B) In cells maintained at the permissive temperature, the ELP 1-G chimeric proteins are targeted to, and resident primarily in, the juxtanuclear Golgi region. C, D) When cells maintained at the permissive temperature were then shifted to the restrictive temperature for 2 h, much of the KDELR-G staining was redistributed to a diffuse ER-like and nuclear envelope pattern that colocalized with the resident ER protein, ERp60. E, F) In cells shifted to the permissive temperature as in C and D, but in the presence of 1 μM BEL, KDELR-G remained colocalized with Man II in the juxtanuclear region. N = nucleus.
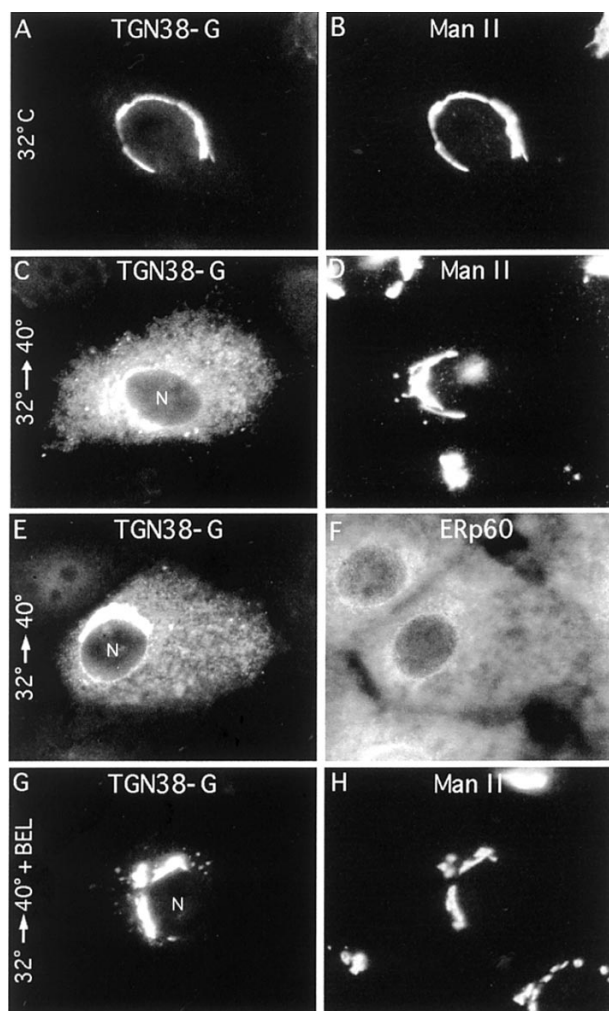

Figure 2: Inhibition of constitutive recycling of TGN38-G from the TGN to the ER by BEL. Double immunofluorescence of TGN38-G (A, C, E, G) and Man II (B, E, H) or ERp60 (F) is shown. A, B) In cells maintained at the permissive temperature, the TGN38-G chimeric proteins are targeted to, and resident primarily in, the juxtanuclear Golgi region in a pattern very similar, but not identical, to Man II. C, D, E, F) When cells maintained at the permissive temperature were then shifted to the restrictive temperature for 2 h, much of the TGN38-G staining was redistributed to a diffuse ER-like and nuclear envelope pattern that colocalized with the resident ER protein, ERp60 (F). G, H) In cells shifted to the permissive temperature as in C and D, but in the presence of 1 μM BEL, TGN38-G remained localized with Man II in the juxtanuclear region. N = nucleus.

We have previously shown that a brief (10 min) pretreatment with BEL did not inhibit the synchronized anterograde trafficking of newly synthesized ts045-VSV-G proteins from ER to Golgi membranes (8). Similar experiments performed with KDELR-G and TGN38-G confirmed that this compound did not acutely affect ER to Golgi trafficking. More specifically, when control cells expressing KDELR-G or TGN38-G were maintained at the restrictive temperature to accumulate these proteins in the ER, treated with BEL (1 μM for 10

min), and then shifted to the permissive temperature in the continuous presence of BEL (for 30 min), KDELR-G and TGN38-G were found, by immunofluorescence microscopy, to have exited the ER and to have moved to downstream compartments where they colocalized with Man II enzymes (data not shown). However, we note that higher concentrations of 2-(p-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082), and other PLA$_2$ antagonists, e.g. N-(p-ainyl-cinnamoyl) anthranilic acid, did inhibit ER-to-Golgi trafficking, although we do not know if this effect was a consequence of a direct or indirect inhibition of COPII-dependent trafficking (32).
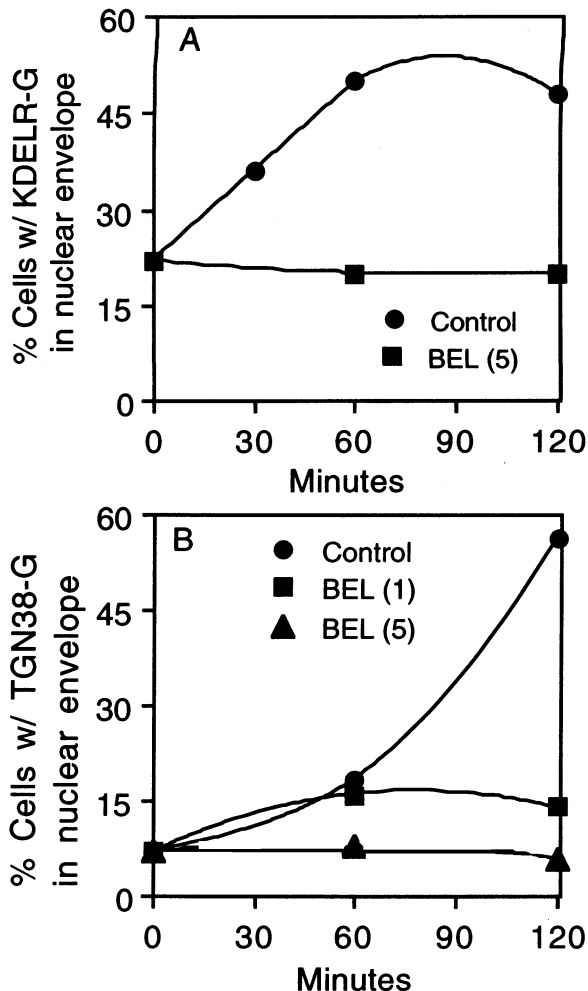

Figure 3: Quantitation of the inhibition of KDELR-G and TGN38-G recycling to the ER. Cells transiently expressing either of KDELR-G (A) and TGN38-G (B) were grown at the permissive temperature and then shifted to the restrictive temperature in the absence or presence of BEL as indicated for 15 min. Cells were then processed for immunofluorescence staining with anti-VSV-G antibodies and the percentage of total transfected cells with staining in the nuclear envelope, as in Figures 1 and 2, was determined. The results represent data obtained from three or four independent experiments.

PLA$_2$ antagonist inhibit recycling of ERGIC-53

The ERGIC-53 protein is a transmembrane lectin that constitutively cycles between the ER, ERGIC, and cis-Golgi elements (15,30). At 15°C, the anterograde and retrograde trafficking of this protein is dramatically impaired such that ERGIC-53 accumulates under these conditions in cis-Golgi and ERGIC elements. Upon warming cells up to 37°C, however, this temperature block is removed and ERGIC-53 can be seen by immunofluorescence and immunoelectron microscopy to exit from the ERGIC and cis-Golgi via membrane tubules and traffic in a retrograde direction back to the ER (13,30,33). Importantly, the so-formed tubules are morphologically and functionally similar to those that constitutively form in cells not previously exposed to low temperature; therefore, the temperature shift to 37°C allows for the direct morphological examination of authentic retrograde transport intermediates. We exploited this system to study the effects of PLA$_2$ inhibitors on retrograde trafficking in the early secretory pathway.

When Clone 9 rat hepatocytes were maintained at 15°C for 3 h, ERGIC-53 was found, as expected, in a collection of juxtanuclear tubules and vesicles that colocalized to a large extent with the cis-Golgi marker, the 10E6 antigen (14) (Figure 4A,B). However, when cells incubated at 15°C for 3 h were subsequently shifted to 37°C for 15 min, ERGIC-53 was found to enter a collection of long membrane tubules and other tubulo-vesicular structures that emanated from the juxtanuclear Golgi region and extended out into the cytoplasm (Figure 4C). In fact, greater than 80% of the examined cells treated in this fashion possessed ERGIC-53 staining in long thin membrane tubules (Figure 5). Interestingly, the cis-Golgi marker, 10E6 was excluded from these retrograde tubules and remained in the Golgi region (Figure 4D). By 30 min, following shift to 37°C, most of the ERGIC-53 staining appeared as a diffuse, ER-like pattern (Figure 4F). Importantly, when cells pre-incubated at 15°C were shifted to 37°C in the presence of ONO-RS-082 (25 µM), ERGIC-53-stained membrane tubules were not observed, ERGIC-53 remained in a juxtanuclear Golgi region, and ERGIC-53 did not redistribute to the ER (compare Figure 4C,D with Figure 4G,H). A time-course of ERGIC-53 tubulation in untreated control and ONO-RS-082-treated cells revealed that half-maximal tubule formation occurred 7.5 min after temperature shift, which was completely abolished by ONO-RS-082 (Figure 5). A similar inhibition of tubule formation was obtained with BEL (25 µM; data not shown).

Microtubules and organelle morphology not disrupted by PLA$_2$ antagonists

Although cytoplasmic microtubules are not required for normal retrograde trafficking (23), they are for efficient BFA-induced recycling. We therefore investigated whether the inhibition in membrane recycling observed in the presence of these PLA$_2$ antagonists was caused by a disruption in microtubule morphological integrity. As we have previously shown (8), the morphology of microtubules was unchanged in cells treated with BEL (1 µM) or ONO-RS-082 (25 µM). In addition,
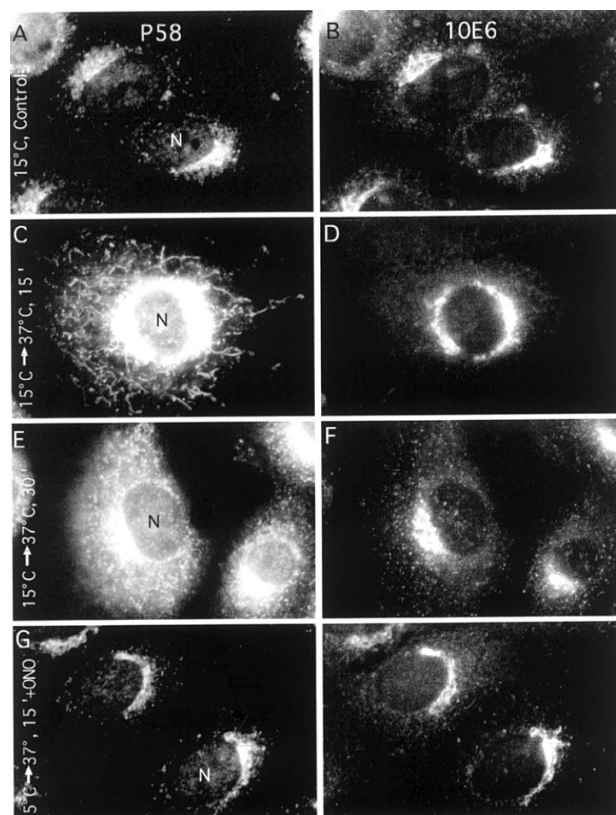
Figure 4: Inhibition of tubule-mediated retrograde trafficking of ERGIC-53 by the PLA$_2$ antagonist, ONO-RS-082 (ONO). Double immunofluorescence localization of ERGIC-53 (A, C, E, G) and a cis-Golgi antigen recognized by monoclonal antibody 10E6 (G, D, F, H) is shown. A, B) When cells were maintained at 15°C, both ERGIC-53 and 10E6 colocalized to a large extent in the juxtanuclear region of the cell. C, D) When cells maintained at 15°C were shifted to 37°C for 15 min, ERGIC-53 entered into an extensive array of membrane tubules that excluded 10E6. E, F) Thirty minutes after shift from 15 to 37°C, ERGIC-53 was found in a diffuse ER-like staining pattern but 10E6 staining was unchanged. G, H) When cells maintained at 15°C were shifted to 37°C for 15 min in the presence of 25 μM ONO-RS-082 (ONO), ERGIC-53 remained in Golgi compartments along with 10E6.

the Golgi complex remained in the juxtanuclear region (see Figures 1 and 4) and did not disperse throughout the cytoplasm as occurs when microtubules are depolymerized with nocodazole (34,35). Finally, a previous immunoelectron microscopic examination of BEL or ONO-RS-082-treated cells revealed that, whereas normal tubular connections between spatially separate stacks are inhibited, the ultrastructural integrity of Golgi stacks was not altered under these conditions, and that the ultrastructure of other cytoplasmic organelles, including lysosomes and prelysosomal compartments, was not significantly disrupted (7). These observations suggest that the antagonists were not simply non-specific disrupters of organelle morphology.

Discussion

We have shown here that antagonists of iPLA$_2$ activity block constitutive retrograde membrane trafficking from the Golgi complex/TGN to the ER as measured by three independent markers: KDELR-G, TGN38-G, and ERGIC-53. BEL was selective in the membrane trafficking events that it inhibited because it failed to acutely affect KDELR-G and TGN38-G transport from ER to cis-Golgi and TGN membranes, respectively, with a short-term treatment (10 min). Similarly, other studies showed that ER-to-Golgi trafficking of vs045 VSV-G was not acutely affected by low concentrations of various membrane-permeant PLA$_2$ antagonists (7). However, as one might predict, long-term treatment with BEL (> 30 min) does appear to inhibit ER-to-Golgi trafficking, probably because molecules needed for vesicle formation could not recycle (unpublished data). Together, these independent observations highlighted the specificity of BEL for some, but not all, membrane trafficking pathways.

All of the relevant molecular targets of BEL are presently unknown; however, a number of lines of evidence show that one major target is the cytosolic iPLA$_2$ enzymes. First, BEL not only possesses more than a 1000-fold specificity for iPLA$_2$ enzymes over their Ca^{2+}-dependent counterparts (1,36,37), but also fails to affect a number of enzyme activities directly involved in cellular arachidonic acid metabolism (38). Second, membrane-free cytosolic extracts of tissue-culture cells treated with BEL and other PLA$_2$ antagonists possess lower levels of iPLA$_2$ activity than their corresponding untreated controls, therefore demonstrating the efficacy of these antagonists under the relevant experimental conditions
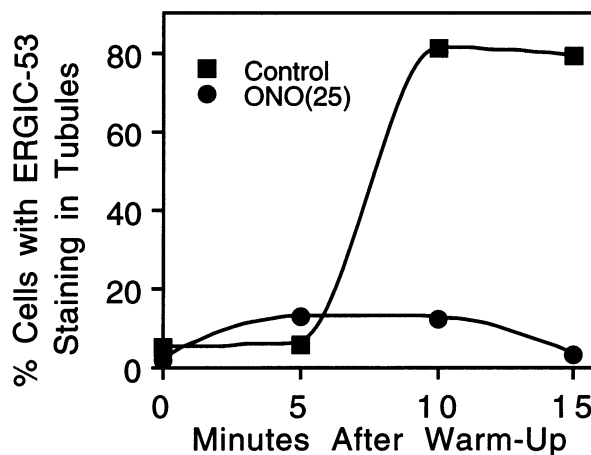

Figure 5: Quantitation of the inhibition of ERGIC-53 tubule formation by the PLA$_2$ antagonist ONO-RS-082 (ONO). Cells were maintained at 15°C for 3 h to accumulate ERGIC-53 in Golgi compartments, shifted to 37°C in the absence or presence of 25 μM ONO, and then at various periods of time after shift, cells were fixed and processed for immunofluorescence staining with ERGIC-53 antibodies. The percentage of total cells exhibiting ERGIC-53-stained tubules (as in Figure 4C) was determined. Each data point represents the average of two independent samples (∼ 200 cells counted/sample).

(7). Finally, the fact that two known antagonists of iPLA$_2$ activities, BEL and ONO-RS-082, both potently inhibit the membrane tubule-mediated recycling of ERGIC-53 proteins from Golgi membranes back to the ERGIC and ER suggests that this recycling block is not merely an inhibitor-specific artifact, but instead, reflects the specificity of these compounds for similar molecular targets, namely, iPLA$_2$ enzymes. Taken together, these pharmacological studies are consistent with the conclusion that iPLA$_2$ enzymes are involved in regulating these membrane trafficking events. These studies do not, however, rule out the possibility that other kinds of BEL-sensitive molecules may also (or alternatively) participate in these processes.

How might antagonist-sensitive iPLA$_2$ enzymes participate in regulating membrane recycling? One possibility is that these enzymes regulate membrane recycling by regulating the formation of Golgi membrane tubules. Over the past 30 years, an assortment of morphological studies have demonstrated the existence of membrane tubules extending from various membrane-bound organelles, and have led to speculations that these tubules may participate in some aspect of membrane trafficking (39–45). For example, membrane tubules of uniform diameter (60–80 nm), but variable length, have been seen to extend from the Golgi complex and TGN outwards into the cytoplasm, and to form direct membrane continuities between otherwise spatially separate cisternal stacks (39,42,43). In the case of the Golgi complex, BFA induced the tubule-mediated retrograde movement of resident enzymes back to the ER (11,13), whereas TGN tubules fused with early endosomes (13,14,46). Studies with BFA suggested, therefore, that membrane tubules may serve as important mediators of trafficking events between various organelles. Support for this idea has recently emerged from time-lapse fluorescence imaging studies, which demonstrated that, at steady-state, membrane tubules are continuously forming and detaching from the Golgi complex (26,27).

Our evidence for iPLA$_2$ activity in tubule-mediated retrograde trafficking came from the use of BFA as a stimulator, which may not precisely reflect normal *in vivo* events. However, the trafficking of ERGIC-53 provided a more direct test because formation of retrograde tubules is not dependent on drug intervention. Our results quite convincingly demonstrated that PLA$_2$ antagonists also inhibited the tubule-mediated retrograde trafficking of ERGIC-53. We also wanted to know if the inhibition of normal recycling by PLA$_2$ antagonists is restricted to ERGIC-53 and found that the constitutive recycling of KDELR-G and TGN38-G were also inhibited. These results suggest that either all of these molecules enter the same PLA$_2$-dependent retrograde pathway, or that multiple PLA$_2$-dependent pathways exit. Also relevant to these studies are previous results demonstrating that nocodazole-induced Golgi mini-stack formation, which is dependent on the constitutive cycling of membranes between the Golgi and ER (19), was also inhibited by PLA$_2$ antagonists (32).

Although ERGIC-53 clearly enters tubules on its way back to the ER (15,30), the exact pathway(s) taken by all recycling molecules is not clear. Studies using GFP-tagged resident Golgi proteins reveal that tubules constantly form, detach, and move into the peripheral cytoplasm where they disappear, presumably because they fuse with the ER (26,27). Clearly, tubule formation is not dependent on coated vesicle proteins because tubulation is stimulated when coat proteins fail to bind, i.e. in BFA-treated cells (12,47). These results suggest that tubules are involved in some aspect of retrograde trafficking. On the other hand, ERGIC-53 contains, in its cytoplasmic tail, the coatomer binding motif K(X)KXX that binds to COPI proteins (48–50). Also, retrograde transport of the KDELR in mammalian cells and the HDELR in yeast has recently been shown to involve COPI proteins (51,52). Finally, a variety of other studies have presented evidence that COPI coated vesicles are involved in moving molecules in a retrograde direction through the Golgi stack (53–57). Therefore, COPI coated vesicles are also involved in Golgi-to-ER retrograde trafficking, at least at the level of intra-cisternal transport.

From the above studies, both tubules and COPI vesicles appear to be involved in retrograde trafficking, but the specific role each plays is unclear. For example, two completely separate parallel pathways from the Golgi to the ER may exist, perhaps one tubule-mediated and one vesicle-mediated. Support for this idea recently came from the identification of a COPI-independent retrograde pathway, which appears to utilize tubules as transport intermediates (22,25). More specifically, studies examining the trafficking of the Golgi-associated GTP-binding protein, rab6, revealed that it moves by retrograde transport from the Golgi to the ER via COPI-independent tubules. This COPI-independent pathway also mediated the retrograde trafficking of certain bacterial toxins (25) and Golgi-associated glycosyl-modifying enzymes (22). Interestingly, ERGIC-53, which contains a COPI-binding motif, also enters into membrane tubules, shown here to be inhibited by PLA$_2$ antagonists, during retrograde trafficking. These results suggest that PLA$_2$-dependent tubules may also be directly or indirectly involved in the COPI-mediated retrograde pathway.

Materials and Methods

Materials

PLA$_2$ inhibitors were obtained from the following sources: 2-(*p*-amyl-cinnaoyl)amino-4-chlorobenzoic acid (ONO-RS-082) and E-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (HELSS, or bromoenol lactone, BEL) were from Biomol Research Laboratories (Plymouth Meeting, PA). All other common reagents were from Sigma Chemical (St Louis, MO).

Plasmids and transient expression procedures for KDELR-G and TGN38-G were as described (20).

Cell culture and treatments

Clone 9 rat hepatocytes were maintained in minimal essential media (MEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in an atmosphere of 95% air, 5% CO$_2$. In a typical

experiment, cells were plated onto glass coverslips (for immunofluorescence) and allowed to grow for 2 days before transfection and/or inhibitor treatment (see below for details). Each inhibitor was freshly prepared as a 1000-fold stock solution in organic solvent (either 100% ethanol or dimethyl sulfoxide as appropriate) and then diluted into MEM just before use. To ensure that microtubule morphology was not disrupted under the various conditions tested, some experiments were performed in the presence of the microtubule stabilizing reagent paclitaxel (see below).

Trafficking assays

We used immunofluorescence to examine the effects of assorted PLA$_2$ inhibitors on the trafficking of chimeric proteins derived from the temperature-sensitive variant (ts045) of vesicular stomatitis virus G (VSV-G) membrane glycoprotein (20). The chimeric proteins were localized using antibodies against the luminal epitopes of VSV-G, which were kindly provided by Dr Bill Balch (Scripps Research Institute). To ensure that the appearance of chimeric proteins back in the ER was due to retrograde trafficking rather than new protein synthesis, cyclohexamide (2 μg/ml) was included in the media after shift to the restrictive temperature. Polyclonal antibodies against the resident ER protein, ERp60, were kindly provided by Dr Michael Green (St Louis University).

In other experiments, we studied the tubule-mediated retrograde trafficking of ERGIC-53 protein from Golgi membranes back to the ER using a temperature-shift protocol as described [(30); see below for details]. Polyclonal anti-rat ERGIC-53 were kindly provided by Dr J. Saraste and cells were processed for immunofluorescence with this antibody as described (15).

Acknowledgments

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